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Molecular and Biochemical Characterization of the Natural Chromosome-Encoded Class A β-Lactamase from *Pseudomonas luteola*▽

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Pseudomonas luteola **(formerly classified as CDC group Ve-1 and named *Chryseomonas luteola*) is an unusual pathogen implicated in rare but serious infections in humans. A novel β-lactamase gene, bla_{LUT-1}, was cloned from the whole-cell DNA of the *P. luteola* clinical isolate LAM, which had a weak narrow-spectrum β-lactam-resistant phenotype, and expressed in *Escherichia coli*. This gene encoded LUT-1, a 296-amino-acid Ambler class A β-lactamase with a pI of 6 and a theoretical molecular mass of 28.9 kDa. The catalytic efficiency of this enzyme was higher for cephalothin, cefuroxime, and cefotaxime than for penicillins. It was found to be 49% to 59% identical to other Ambler class A β-lactamases from *Burkholderia* sp. (PenA to PenL), *Ralstonia eutropha* (REUT), *Citrobacter sedlakii* (SED-1), *Serratia fonticola* (FONA and SFC-1), *Klebsiella* sp. (KPC and OXY), and CTX-M extended-spectrum β-lactamases. No gene homologous to the regulatory *ampR* genes of class A β-lactamases was found in the vicinity of the bla_{LUT-1} gene. The entire bla_{LUT-1} coding region was amplified by PCR and sequenced in five other genetically unrelated *P. luteola* strains (including the *P. luteola* type strain). A new variant of bla_{LUT-1} was found for each strain. These genes (named bla_{LUT-2} to bla_{LUT-6}) had nucleotide sequences 98.1 to 99.5% identical to that of bla_{LUT-1} and differing from this gene by two to four nonsynonymous single nucleotide polymorphisms. The bla_{LUT} gene was located on a 700- to 800-kb chromosomal I-CeuI fragment, the precise size of this fragment depending on the *P. luteola* strain.**

Pseudomonas luteola (formely known as CDC group Ve-1 or *Chryseomonas luteola*) is a motile, strictly aerobic, gram-negative rod, producing a distinct yellow-orange pigment (4). This organism is nonfermentative, oxidase negative, and catalase positive. *P. luteola* has been isolated from many sources in nature (water, soil, and damp environments) and is considered to be a saprophyte or commensal organism only rarely pathogenic to humans (11, 19). Clinical infections due to this microorganism have rarely been reported (fewer than 25 cases) and have mostly presented as septicemia, meningitis, peritonitis, endocarditis, and ulcer infections, usually in association with surgical operations or the use of catheters or prostheses (11, 13–15, 17, 19, 20, 24, 36, 42). It has been suggested that this organism is likely to become more frequent as a nosocomial pathogen (19). The clinical isolates of *P. luteola* have generally been shown to be susceptible to extended-spectrum cephalosporins (ESC), aminoglycosides, and fluoroquinolones (11, 20, 36, 42). In most studies in which isolates were tested with a large panel of β-lactam antibiotics, resistance to original-spectrum and broad-spectrum cephalosporins was observed, whereas susceptibility to penicillins was variable (5, 13–15, 17, 20, 36). This β-lactam resistance phenotype suggests that this microbe may produce a natural β-lactamase.

We report here the cloning and sequencing of the bla_{LUT-1} gene, encoding the class A β-lactamase of the *P. luteola* clinical isolate LAM, which was isolated in January 2002 from a blood culture from a patient with an infected indwelling catheter. We investigated the biochemical characteristics of LUT-1. The presence, nucleotide diversity, and location of the bla_{LUT} gene were studied in five other genetically unrelated *P. luteola* strains.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. *P. luteola* was identified with the API-20 NE system (bioMérieux, Marcy-l'Etoile, France) and by sequencing PCR-amplified *rrs* (16S rRNA gene) and *rpoB* (RNA polymerase beta subunit), as previously described (2).

Antimicrobial susceptibility testing. Antibiotic susceptibility was assessed by the disk diffusion method for 32 antimicrobial drugs (Bio-Rad, Marnes La Coquette, France), as previously described (43). The MIC of each β-lactam antibiotic was determined by Etest (AB Biodisk, Solna, Sweden). Susceptibility was classified according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (CA-SFM) (http://www.sfm.asso.fr/nouv/general.php?pa=2). *Escherichia coli* ATCC 25922 was used as the control for disk diffusion analyses and MIC determinations.

Cloning experiments and analysis of recombinant plasmids. Genomic DNA of the *P. luteola* strain LAM was partially digested with Sau3AI restriction enzyme, ligated into the BamHI-restricted phagemid pBK-CMV, and electroporated into *E. coli* strain DH10B, as previously described (34). Antibiotic-resistant colonies were selected on Mueller-Hinton (MH) agar (Bio-Rad) containing kanamycin (30 μg/ml) and cefamandole (5 μg/ml).

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TABLE 1. Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Description	Source
Strains		
P. luteola		
LAM	Clinical isolate (blood/ICVL infection), 2002, Paris, France; resistant to CEF, FAM, FOX, CHL, SUL, TMP, SXT, NAL	HSL
02-5971	Clinical isolate (peritoneal fluid/peritoneal mixed bacterial infection), 2002, Lons-Le-Saunier, France; resistant to CEF, FAM, FOX, TMP, SXT, NAL	BBPE, IP
03-5093	Clinical isolate (blood/prosthetic valve endocarditis), 2003, Arles, France; resistant to CEF, FAM, FOX, TMP, SXT, NAL	BBPE, IP
04-8684	Clinical isolate (blood/IVAD infection), 2004, Annonay, France; resistant to CEF, FAM, FOX, TMP, SXT, NAL	BBPE, IP
HEGP	Hospital environment isolate, 2002, Paris, France; resistant to CEF, FAM, FOX, TMP, SXT, NAL	HEGP
CIP 102995^T	Type strain (human wound); resistant to CEF, FAM, FOX, TMP, SXT, NAL	CIP, IP
E. coli		
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 ara*Δ*139* Δ(*ara leu*)*7697 galU galK-rpsL nupG tonA*	Invitrogen
ATCC 25922		CIP, IP
Plasmids		
pBK-CMV phagemid	Neo^r Kan^r	Boehringer-Mannheim
pBK-L3	1.5-kb Sau3AI DNA fragment from *P. luteola* LAM that contained bla_{LUT-1} in the BamHI site of pBK-CMV	Present study

[a] Abbreviations: CEF, cephalothin; FAM, cefamandole; FOX, cefoxitin; CHL, chloramphenicol; SUL, sulfonamides; TMP, trimethoprim; SXT, sulfamethoxazole-trimethoprim; NAL, nalidixic acid; ICVL, indwelling central vascular line; IVAD, implantable venous access device; IP, Institut Pasteur; CIP, Collection de l'Institut Pasteur; HEGP, Hôpital Européen Georges Pompidou; HSL, Hôpital Saint-Louis; BBPE, Unité de Biodiversité des Bactéries Pathogènes Emergentes.

The 1.5-kb cloned DNA fragment from the recombinant plasmid pBK-L3 was sequenced on both strands by Cogenics (Meylan, France). Analyses of nucleotide sequences and deduced amino acid sequences were performed with EditSeq and Megalign software (DNAstar, Madison, WI). The BLAST programs available from the NCBI were used for database searches (http://www.ncbi.nlm.nih.gov/BLAST/).

We searched for a divergently transcribed regulator gene upstream for the bla_{LUT-1} gene in various recombinant plasmids shown by PCR to contain the longest DNA sequences upstream from the bla_{LUT-1} gene. The primers used were either T3 or T7 (binding to the multicloning site of pBK-CMV) and REG (5′-CTTTTGTGACTTGAGGAGATCGCA-3′) (binding 300 bp upstream from the ATG initiation codon of the LUT-1 gene). The amplification conditions were as described below, except that an annealing temperature of 47°C was used.

Isoelectric focusing and β-lactamase preparation. Isoelectric focusing was performed with polyacrylamide gels containing ampholines with a pH range of 3.5 to 10, as previously described (7, 38). β-Lactamases of known pIs (in parentheses) were used as standards: TEM-1 (5.4), TEM-2 (5.6), TEM-6 (5.9), TEM-24 (6.5), and TEM-28 (6.1).

LUT-1-producing *E. coli* DH5α(pBK-L3) was grown in 6 liters of 2× yeast-tryptone broth supplemented with 20 μg/ml kanamycin and 32 μg/ml amoxicillin for 18 h at 37°C. The β-lactamase LUT-1 was purified as previously described (8, 37), by ion-exchange chromatography with a Q Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden) and gel filtration chromatography with a Superose 12 column (Amersham Pharmacia Biotech). Total protein concentration was estimated with the Bio-Rad protein assay (Bio-Rad, Richmond, CA) with bovine serum albumin (Sigma Chemical Co., St. Louis, MO) used as the standard. The purity of LUT-1 extracts was estimated as previously described (9), by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie blue R-250 (Sigma Chemical Co.).

Determination of kinetic constants for β-lactamase activity. The kinetic constants K_m and k_{cat} for β-lactamases were obtained by a computerized microacidimetric method, as previously described (26). The concentrations of the inhibitors (clavulanate and tazobactam) required to inhibit enzyme activity by 50% (IC_{50}s) were determined with penicillin G (9). Penicillin G (100 mM) was used as the reporter substrate for IC_{50} monitoring. The kinetic constants were determined three times. The coefficients of variation did not exceed 15%.

PFGE typing. The genetic diversity of *P. luteola* was assessed by pulsed-field gel electrophoresis (PFGE) of genomic DNA digested with XbaI or SpeI (Roche), as previously described (43).

PCR amplification and sequencing of the bla_{LUT} gene in *P. luteola*. The entire coding region of the bla_{LUT} gene of all *P. luteola* strains was amplified by PCR, using the primers UpPlut (5′-ACCGTCTAGGCTGCTACTTCA-3′) and LoPlut (5′-CCGCTGCGCATGAGCGTA-3′), binding 200 bp upstream from the ATG initiation codon of LUT-1 and 10 bp downstream from the stop codon, respectively. The PCR products (1,100 kb) were sequenced at the Plateforme de Génotypage des Pathogènes et Santé Publique, PF8 (Institut Pasteur).

Phylogenetic analysis of the amino acid sequences. The ClustalW program (http://infobiogen.fr) was used to align the amino acid sequences obtained (40). Phylogenetic analysis was carried out with the bioinformatics tool TOPALi v2.5 (28, 29). A phylogenic tree was constructed by the Bayesian method, as implemented in the MRBAYES program (22). LUT-1 was compared with 37 class A β-lactamases. The consensus tree calculated by MRBAYES was imported into MEGA4 for the purposes of displaying and printing the tree (25, 39).

Resistance transfer determination. Conjugation and transformation experiments were carried out on the *P. luteola* LAM isolate, as previously described (43). The β-lactam antibiotic used for selection was cefotaxime with a final concentration of 0.25 μg/ml.

Chromosomal location of bla_{LUT} genes, as determined by PFGE–I-CeuI. For determination of the chromosomal location of the bla_{LUT-1} gene, we digested agarose plugs, prepared as described previously, with the I-CeuI endonuclease (New England Biolabs, Beverly, MA) (27). I-CeuI restriction fragments were subjected to Southern blot hybridization with a PCR-generated probe for the bla_{LUT-1} gene and with an *rrs* (16S rRNA gene) probe, as described above. Hybridization, labeling, and detection were performed according to the manufacturer's recommendations, using a nonradioactive enhanced chemiluminescence kit (ECL; GE Healthcare, United Kingdom).

Nucleotide sequence accession numbers. The nucleotide sequences of the bla_{LUT-1} to bla_{LUT-6} genes of the *P. luteola* strains have been deposited in the GenBank database under accession numbers AY695112, FJ750572, FJ750573, FJ750574, FJ750575, and FJ750576, respectively.

RESULTS AND DISCUSSION

Antimicrobial susceptibility of *P. luteola*. The disk diffusion method showed the five *P. luteola* isolates and the type strain to be resistant to cephalothin, cefamandole, cefoxitin (resistance

TABLE 2. MICs of β-lactams for *P. luteola* and *E. coli* strains

β-Lactam[a]	MIC (μg/ml) for strain(s):			
	P. luteola LAM (LUT-1)	Other *P. luteola* strains (LUT-2 to LUT-6)	*E. coli* DH10B(pBK-L3) (LUT-1)	*E. coli* DH10B
Amoxicillin	4	4–8	128	4
Amoxicillin-CLA[b]	1	1–2	4	4
Ticarcillin	8	8–16	256	2
Ticarcillin-CLA[c]	4	2–4	16	2
Piperacillin	2	2–4	8	1
Piperacillin-TZB[d]	1	1–2	1	1
Cephalothin	64	128–256	32	4
Cefoxitin	32	32–128	4	4
Ceftazidime	0.5	0.5–1	1	0.125
Ceftazidime-CLA[e]	0.25	0.25–0.5	0.5	0.25
Cefotaxime	1	2–8	1	<0.06
Cefotaxime-CLA[e]	1	1–>1	0.06	<0.06
Cefepime	0.5	1–4	0.5	0.06
Aztreonam	2	4–8	2	<0.06
Imipenem	0.25	0.25–0.5	0.5	0.5

[a] CLA, clavulanic acid; TZB, tazobactam.
[b] Amoxicillin-CLA ratio, 2:1.
[c] CLA, 2 μg/ml.
[d] TZB, 4 μg/ml.
[e] CLA, 4 μg/ml.

or intermediate susceptibility), nalidixic acid, trimethoprim, and trimethoprim-sulfamethoxazole. All strains were susceptible to ciprofloxacin and aminoglycosides. Additional resistance to sulfonamides and chloramphenicol was observed only in the LAM isolate. The MICs of the β-lactams determined by Etest are shown in Table 2. The addition of clavulanic acid slightly decreased (by a factor of 2 to 4) the MICs of amoxicillin, ticarcillin, and piperacillin. The MICs of the ESC and also aztreonam were slightly affected within the susceptible or intermediate range. Only the cefotaxime MICs of some *P. luteola* strains were in the resistant (>2-μg/ml) range according to the guidelines of the CA-SFM. By using the Etest ESBL cefepime/cefepime plus clavulanic acid strips, a deformation of the cefepime inhibition ellipse was observed for two strains with the highest MICs of cefepime (isolate 03-5093 and type strain CIP 102995^T). The MICs of nalidixic acid and ciprofloxacin ranged from 16 to >256 μg/ml and from 0.032 to 0.25 μg/ml, respectively.

Cloning and sequence analysis of the *bla* gene from *P. luteola*. Partially Sau3AI-digested DNA from the *P. luteola* clinical isolate LAM was inserted into the BamHI site of pBK-CMV. Ten *E. coli* DHB10B recombinant clones were obtained after selection on kanamycin and cefamandole (5 μg/ml). The inserts of the recombinant plasmids were between 1.5 and 3.6 kb in size. The pBK-L3 plasmid, which had a 1.5-kb insert, was selected for sequence analysis. An open reading frame (ORF) of 891 bp, preceded by a putative promoter region (339 bp), was identified and shown to encode a 296-amino-acid sequence. These nucleotide and amino acid sequences were absent from databases. However, the deduced protein had amino acid motifs typical of Ambler class A β-lactamases (^{70}SXXK^{73}, ^{130}SDN^{132}, ^{166}EXXXN^{170}, and ^{234}KTG^{236}) (3). We therefore named this putative novel class A β-lactamase and the corresponding gene LUT-1 and bla_{LUT-1}, respectively. We used the SignalP 3.0 server (available at: http://www.cbs.dtu.dk/services/SignalP/) to determine whether this protein had a putative signal peptide. A putative cleavage site was identified between the 27th and 28th amino acids of the N-terminal region, giving a putative mature protein with a theoretical molecular mass of 28.9 kDa. A phylogenic study was carried out to assess the relationship between LUT-1 and its closest relatives and between this enzyme and members of the major lineages of class A β-lactamases (Fig. 1). The predicted LUT-1 protein showed similarities to several other chromosome-encoded class A β-lactamases identified in beta- and gammaproteobacteria. Figure 2 shows an alignment of the amino acid sequence of LUT-1 with representative members of the various branches of naturally occurring and acquired class A β-lactamases displaying similarity to LUT-1. The LUT-1 β-lactamase was 53 to 59% similar to the chromosomal β-lactamases of *Burkholderia cepacia* complex (Pen-A to Pen-L), 56% identical to that of *Ralstonia eutropha* (REUT), 54% identical to that of *Citrobacter sedlakii* (SED-1), 52% identical to that of *Serratia fonticola* (FONA-5), and 51% identical to that of *Klebsiella oxytoca* (OXY-5) (16, 33, 34, 44). LUT-1 also showed similarities with acquired β-lactamases such as KPC-7 from *Klebsiella pneumoniae* and SFC-1 from *S. fonticola* (class A carbapenemases) (21, 30, 44). Interestingly, the LUT-1 β-lactamase was also 49 to 52% identical to members of the extended-spectrum β-lactamase (ESBL) CTX-M family (6).

We identified no putative Lys-R-type regulator upstream from the ORF encoding LUT-1 in pBK-L3. The upstream regions of the bla_{LUT-1} gene were amplified from nine other recombinant plasmids with the T3 and REG primers. The three plasmids containing the longest regions (750 bp, 600 bp, and 500 bp, respectively) were sequenced, but no regulator gene was identified. However, our results do not completely rule out the possibility that there is a regulator gene.

Properties of the LUT-1 β-lactamase. The recombinant *E. coli*(pBK-L3) clone had a β-lactam resistance phenotype different from that of the parental strain *P. luteola* LAM (Table 2). In *E. coli*, LUT-1 conferred resistance to amoxicillin and ticarcillin and an intermediate level of susceptibility to piperacillin and cephalothin. However, *E. coli*(pBK-L3) remained susceptible to cefoxitin, ESC, and imipenem. The β-lactamase inhibitors lowered the MICs of amoxicillin, ticarcillin, piperacillin, and cefotaxime by factors of 8 to 32. By using the Etest ESBL cefepime/cefepime plus clavulanic acid strips, a deformation of the cefepime inhibition ellipse was observed indicative of ESBLs. The discrepancy between *P. luteola* LAM and *E. coli*(pBK-L3) β-lactam resistance phenotypes may be due to the production of larger amounts of enzyme when the gene is expressed on a high-copy-number plasmid in *E. coli*. β-Lactam resistance phenotypes of the clinical *P. luteola* isolates (i.e., susceptibility to hydrolyzable penicillins) suggested a small amount of LUT. This hypothesis is strengthened by the kinetic parameters of LUT described below. Efflux mechanisms and/or differences in outer membrane permeability may also alter periplasmic β-lactam concentrations, thereby affecting apparent enzyme activity.

Both *P. luteola* isolate LAM and *E. coli*(pBK-L3) produced a single β-lactamase with an isoelectric point (pI) of approximately 6 (data not shown), consistent with the calculated theoretical pI. The purified LUT-1 protein appeared on SDS-polyacrylamide gels as a single band (≥98% pure) of

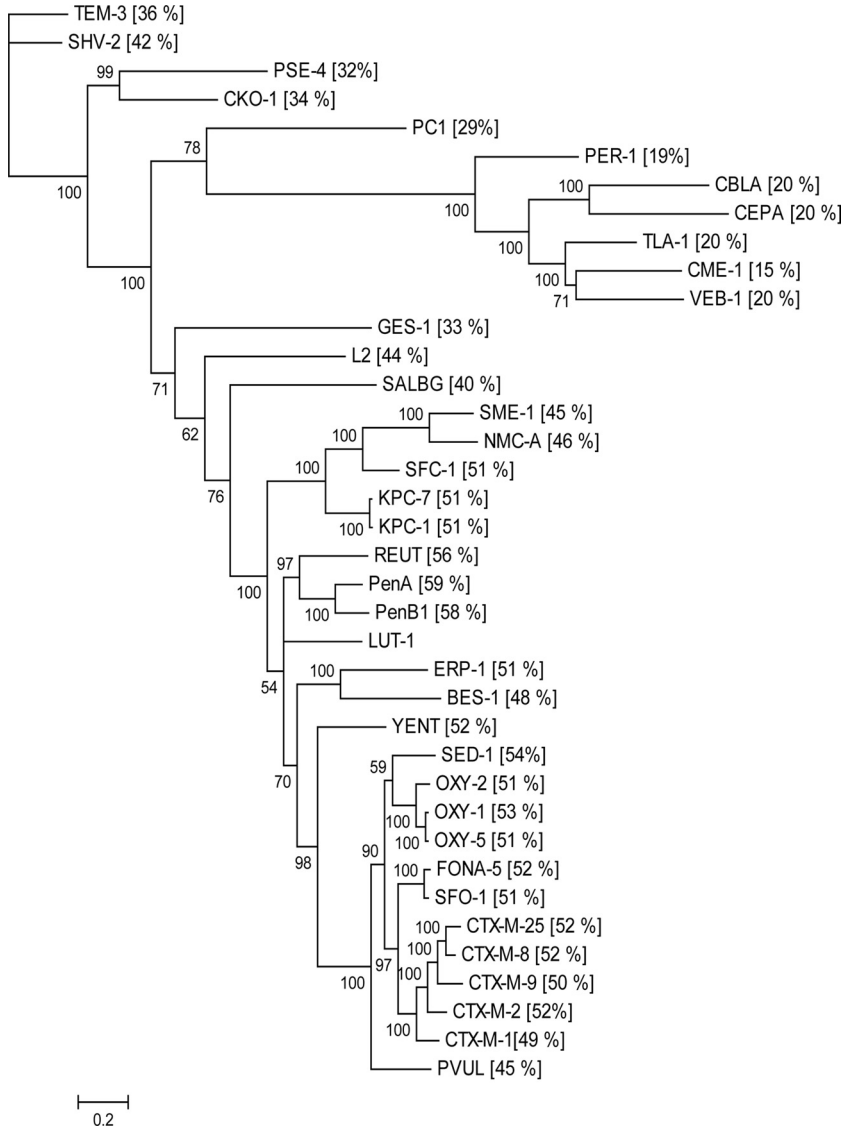

FIG. 1. Phylogeny of the amino acid sequences of LUT-1 and 37 other class A β-lactamases constructed with TOPALi v2.5 (28, 29). Branch lengths are drawn to scale and are proportional to the numbers of amino acid changes. The percentages at the branch points refer to the numbers of times that particular nodes were found in 10,000 bootstrap replications. The distance along the vertical axis has no significance. The amino acid identity of each β-lactamase to the amino acid sequence of LUT-1 from *P. luteola* is indicated in parentheses. The acquired β-lactamases of Gram-negative organisms (GenBank accession numbers are indicated in parentheses) are TEM-3 (X64525), SHV-2 (L47119), PSE-4 (J05162), PER-1 (Z21957), TLA-1 (AAD37403), VEB-1 (AF010416), BES-1 (AF234999), CTX-M-1 (X92506), CTX-M-2 (X92507), CTX-M-8 (AF189721), CTX-M-25 (AAM70498), CTX-M-9 (AAF05311), GES-1 (AF156486), KPC-1 (AF297554), KPC-7 (EU729727), PC-1 (X04121), SFO-1 (AB003148), and SFC-1 (AY354402). The others are naturally occurring β-lactamases, such as CKO-1 from *Citrobacter koseri* (AF477396); CME-1 from *Chryseobacterium meningosepticum* (AJ006275); CEPA from *Bacteroides fragilis* (U05888); CBLA from *Bacteroides uniformis* (L08472); SALBG from *Streptomyces albus* (M28303); PenA from *Burkholderia multivorans* (AAB53622); PenB1 from *Burkholderia cenocepacia* (EU872211); NMC-A from *Enterobacter cloacae* (Z21956); SME-1 from *Serratia marcescens* (Z28968); REUT from *Ralstonia eutropha* (YP_295893); YENT from *Yersinia enterocolitica* (X57074); SED-1 from *Citrobacter sedlakii* (AF321608); ERP-1 from *Erwinia persicina* (AAL86999); PVUL from *Proteus vulgaris* (X80128); OXY-1, OXY-2, and OXY-5 from *Klebsiella oxytoca* (Z30177, Z49084, and AJ871868, respectively); FONA-5 from *Serratia fonticola* (AJ251243); and L2 from *Stenotrophomonas maltophilia* (CAB63490).

approximately 29 kDa (data not shown). Kinetic parameters indicated that the LUT-1 enzyme had a broad substrate profile including penicillins and cephalosporins, such as cephalothin, cefuroxime, and cefotaxime in particular (Table 3). As observed for CTX-M, LUT-1 displayed stronger hydrolytic activity against cefotaxime than against ceftazidime or aztreonam (k_{cat}, 452 versus 1.3 or 1.5 s^{-1}, respectively). It was inhibited by low concentrations of clavulanic acid (IC_{50}, 36 nM) and tazobactam (IC_{50}, 40 nM). Given its susceptibility to clavulanate and its ability to cleave ESC, we can classify LUT-1 as a member of the 2e group in the functional classification of β-lactamases (10). Particular residues of LUT-1 such as Ser104, Thr167, Thr237, and Arg276, identified as important positions for the action of other ESBLs (TEMs and CTX-Ms),

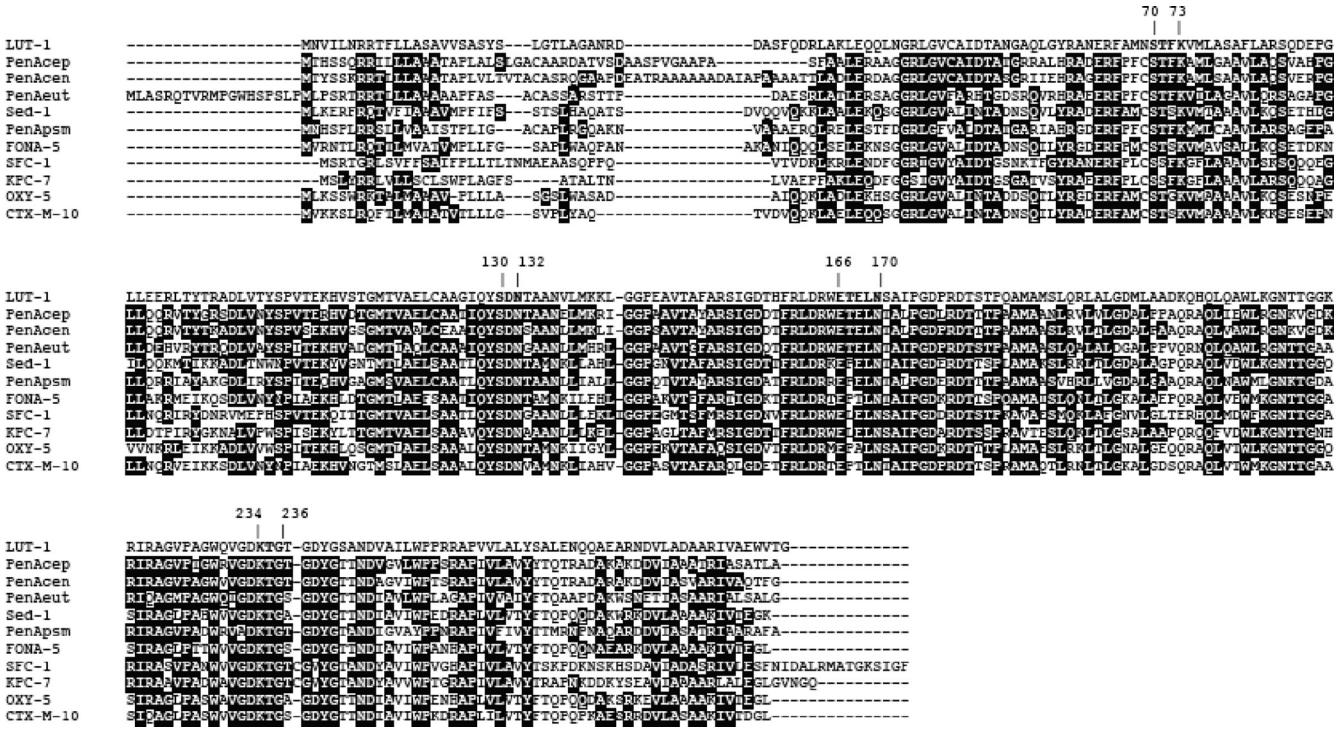

FIG. 2. Alignment of the LUT-1 β-lactamase amino acid sequence from *P. luteola* with those of other class A β-lactamases. Naturally occurring β-lactamases: PenA from *Burkholderia multivorans* 249 (41), PenB1 from *B. cenocepacia* (GenBank accession no. EU872211), REUT from *Ralstonia eutropha* (GenBank accession no.Q470Y4), SED-1 from *Citrobacter sedlakii* (33), FONA-5 from *Serratia fonticola* (GenBank accession no. Q9RIQ8), and OXY-5 from *K. oxytoca* (16). Acquired β-lactamases: SFC-1 from *Serratia fonticola* (21), KPC-7 from *Klebsiella pneumoniae* (GenBank accession no. ACE62798), and CTX-M-2 extended-spectrum β-lactamase (6). The positions of typical class A β-lactamase amino acids are indicated according to the standard numbering scheme of Ambler et al. (3).

may be involved in the activity of LUT-1 against oxyiminocephalosporins (12, 18, 23, 31, 32, 35).

The level of hydrolysis of oxyiminocephalosporins observed, particularly for cefotaxime, was surprisingly high given their low MICs (Table 2). However, low levels of expression in *P. luteola* LAM may be responsible for these low MICs, suggesting a possible chromosomal location for the bla_{LUT} gene. The presence of the natural promoter region (in silico analysis [data not shown]) may partly account for the low level of resistance observed in *E. coli*(pBK-L3).

No biochemical characterization of the five variants of the LUT-1 β-lactamase (LUT-2 to LUT-6) identified on the basis of the bla_{LUT} gene sequences (see below) was performed. The

TABLE 3. Substrate profile of the LUT-1 β-lactamase

β-Lactam	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
Penicillin G	31	8	3.87
Amoxicillin	21	20	1.05
Ticarcillin	5	14	0.36
Piperacillin	8	7	1.14
Cephalothin	543	21	25.86
Cefuroxime	286	24	11.91
Cefotaxime	452	86	5.25
Ceftazidime	1.3	35	0.04
Aztreonam	1.5	40	0.04

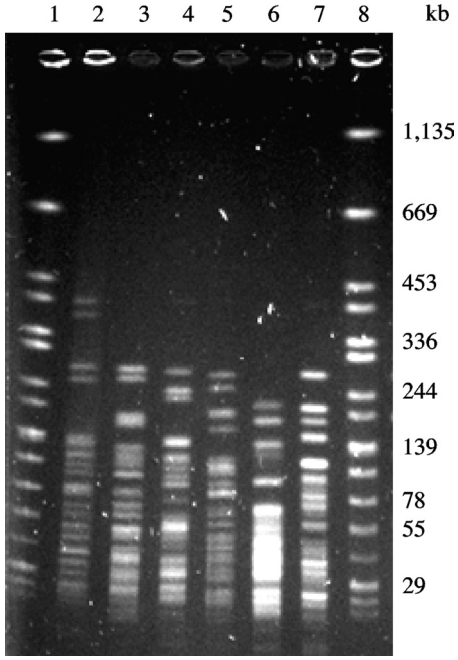

FIG. 3. Pulsed-field gel electrophoresis of SpeI-digested genomic DNA from *P. luteola*. Lanes 1 and 8, XbaI-digested genomic DNA from *Salmonella enterica* serotype Braenderup H9812, used to provide molecular size markers (band sizes in kilobases); lane 2, clinical isolate LAM; lane 3, type strain CIP 1102995^T; lane 4, clinical isolate 02-5971; lane 5, clinical isolate 03-5093; lane 6, clinical isolate 04-8684; lane 7, clinical isolate HEGP.

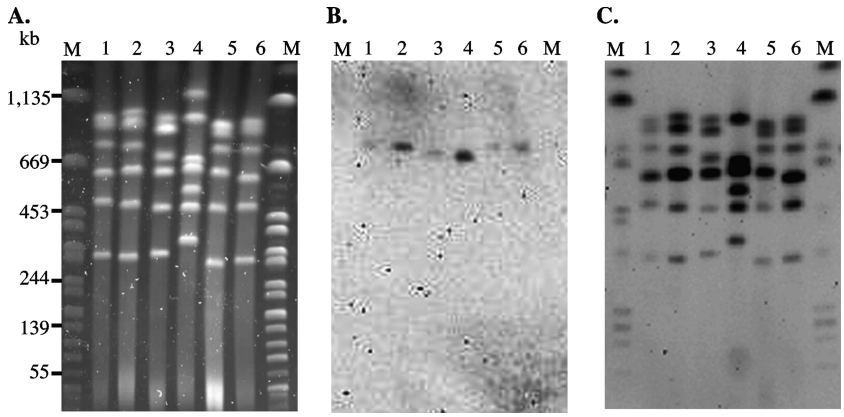

FIG. 4. Chromosomal location of the bla_{LUT-1} gene. (A) PFGE separation of I-CeuI-digested DNA from *P. luteola*. Lane 1, clinical isolate LAM; lane 2, type strain CIP 1102995^T; lane 3, clinical isolate 02-5971; lane 4, clinical isolate 03-5093; lane 5, clinical isolate 04-8684; lane 6, clinical isolate HEGP; lanes M, XbaI-digested genomic DNA from *S. enterica* serotype Braenderup H9812 used to provide molecular size markers (band sizes in kilobases). (B) Southern blot hybridization with the bla_{LUT-1} probe. (C) Southern blot hybridization with the *rrs* (16S rRNA gene) probe. For panels B and C, lanes are as described for panel A.

effects of the amino acid substitutions on the hydrolytic profile of the protein therefore remain unclear.

Diversity of the bla_{LUT} gene in *P. luteola* strains. We used PCR with the UpPlut and LoPlut primers to determine whether the bla_{LUT-1} gene was present in the six *P. luteola* strains and in type strains of *P. aeruginosa*, *B. cepacia*, *S. fonticola*, *Yersinia enterocolitica*, *C. sedlakii*, and *K. oxytoca*. All the *P. luteola* strains yielded a PCR product of the expected size (1,100 bp). No amplification was observed for the other species. However, the specificity of the PCR requires confirmation through testing in several other rare *Pseudomonas* species closely related to *P. luteola* in the phylogenetic tree (e.g., *P. anguilliseptica*, *P. pertucinogena*, and *P. lundensis*) (1). Sequencing of the PCR products on both strands of the DNA revealed that the six bla_{LUT} genes had nucleotide sequences 98.1 to 99.5% identical to that of the bla_{LUT-1} gene. Two to four nonsynonymous single nucleotide polymorphisms with respect to the LUT-1 β-lactamase were observed. These five variants of the bla_{LUT-1} gene were named bla_{LUT-2} to bla_{LUT-6} and deposited in the GenBank database.

Genomic diversity of *P. luteola* strains. The genomic diversity of the six *P. luteola* strains was assessed by PFGE of XbaI- or SpeI-digested whole-cell DNA. The XbaI enzyme did not appear suitable for this species due to the presence of numerous compressed bands of small molecular size (in the range of 0 to 100 kb) (data not shown). However, the *P. luteola* strains were found to be genetically unrelated, as each strain harbored an SpeI profile differing from those of the other strains by at least 7 bands (Fig. 3).

Chromosomal location of bla_{LUT} genes in *P. luteola*. β-Lactam resistance could not be transferred by conjugation or by electroporation from the *P. luteola* LAM isolate to *E. coli* by using cefotaxime at 0.25 μg/ml for selection. These results suggested that the bla_{LUT-1} gene might be located on the chromosome. We tested this hypothesis, by digesting high-molecular-weight *P. luteola* DNAs embedded in agarose plugs with I-CeuI, separating the digestion products by PFGE and Southern blotting, and hybridizing them with a PCR-generated bla_{LUT-1} probe and an *rrs* (16S rRNA gene) probe. The bla_{LUT} genes were assigned to a single large I-CeuI fragment, between 700 and 800 kb in size, depending on the strain, which also hybridized with the *rrs* probe (Fig. 4B and C). All the *P. luteola* strains harbored 6 chromosomal I-CeuI fragments which hybridized with the *rrs* probe (Fig. 4C). These results demonstrated a chromosomal location for bla_{LUT} genes.

In conclusion, we have identified LUT-1, a class A β-lactamase naturally occurring in *P. luteola*. *P. luteola* has a weak narrow-spectrum β-lactam-resistant phenotype, but this environmental species may act as a reservoir of β-lactam resistance determinants. Provided that these findings are confirmed with a larger number of *P. luteola* (sensitivity) and *Pseudomonas* sp. (specificity) isolates, two practical applications of this study would be the use of the bla_{LUT} gene as a molecular identification marker for *P. luteola* species and the use of the DNA sequence microvariation of this gene as an alternative to PFGE for strain differentiation during investigations of outbreaks.

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